

Amendments to the Specification:

Please replace the paragraph beginning on page 1, line 2, with the following amended paragraph:

This application is a continuation of U.S. Application No. 09/501,708 filed February 10, 2000 which, claims the benefit of U.S. Provisional Application Numbers 60/119,571 filed 2/10/1999, now abandoned, and 60/139,172 filed 6/15/99, now abandoned, ~~all~~ each of which ~~are~~ is hereby incorporated herein by reference in ~~their entireties~~ its entirety.

Please replace the paragraph beginning on page 2, line 30, with the following amended paragraph:

In one embodiment, the purified enzyme is fewer than 450 amino acids in length, comprising a polypeptide having the amino acid sequence SEQ ID NO: 70 [63-452]. In preferred embodiments, the purified protein exists in a variety of "truncated forms" relative to the proenzyme referred to herein as SEQ ID NO: 2 [1-501], such as forms having amino acid sequences SEQ ID NO: 70 [63-452], SEQ ID NO: 69 [63-501], SEQ ID NO: 67 [58-501], SEQ ID NO: 68 [58-452], SEQ ID NO: 58 [46-452], SEQ ID NO: 74 [22-452], ~~SEQ ID NO: 58 [46-452]~~. More generally, it has been found that particularly useful forms of the enzyme, particularly with regard to the crystallization studies described herein, are characterized by an N-terminus at position 46 with respect to SEQ ID NO: 2 and a C-terminus between positions 452 and 470 with respect to SEQ ID NO: 2. and more particularly, by an N-terminus at position 22 with respect to SEQ ID NO: 2 and a C-terminus between positions 452 and 470 with respect to SEQ ID NO: 2. These forms are considered to be cleaved in the transmembrane "anchor" domain. Other particularly useful purified forms of the enzyme include: SEQ ID NO: 43[46-501], SEQ ID NO: 66 [22-501], and SEQ ID NO: 2 [1-501]. More generally, it is appreciated that useful forms of the enzyme have an N-terminal residue corresponding to a residue selected from the group consisting of residues 22,46,58 and 63 with respect to SEQ ID NO: 2 and a C-terminus selected from a residue between positions 452 and 501 with respect to SEQ ID NO: 2 or a C-terminus between residue positions 452 and 470 with respect to SEQ ID NO: 2. Also described herein are forms of enzyme isolated from a mouse, exemplified by SEQ ID NO: 65.

Please replace the paragraph beginning on page 3, line 18, with the following amended paragraph:

This invention is further directed to a crystalline protein composition formed from a purified β -secretase protein, such as the various protein compositions described above. According to one embodiment, the purified protein is characterized by an ability to bind to the β -secretase inhibitor substrate P10-P4'sta D \rightarrow V which is at least equal to an ability exhibited by a protein having the amino acid sequence ~~SEQ ID NO: 70~~ SEQ ID NO: 71 [46-419], when the proteins are tested for binding to said substrate under the same conditions. According to another embodiment, the purified protein forming the crystallization composition is characterized by a binding affinity for the β -secretase inhibitor substrate SEQ ID NO: 72 (P10-P4'sta D \rightarrow V) which is at least 1/100 of an affinity exhibited by a protein having the amino acid sequence SEQ ID NO: 43 [46-501], when said proteins are tested for binding to said substrate under the same conditions. Proteins forming the crystalline composition may be glycosylated or deglycosylated.

Please replace the paragraph beginning on page 8, line 14, with the following amended paragraph:

FIG. 5 shows the full length amino acid sequence of β -secretase 1-501 (SEQ ID NO: 2), including the ORF which encodes it (SEQ ID NO: 1), with certain features indicated, such as "active-D" sites indicating the aspartic acid active catalytic sites, a transmembrane region commencing at position 453, as well as leader ("Signal") sequence (residues 1-21; SEQ ID NO: 46) and the putative pro region (residues 22-45; SEQ ID NO: 47) and where the polynucleotide region corresponding the proenzyme region corresponding to amino acids 46-501 (SEQ ID NO: 43) (~~nt 135-1503~~) is shown as ~~SEQ ID NO: 44~~ SEQ ID NO: 42 (nt 135-1503 of SEQ ID NO: 1) and contains an internal peptide region (SEQ ID NO: 56) and a transmembrane region (SEQ ID NO: 62).

Please replace the paragraphs at page 10, lines 29-30 with the following amended paragraphs:

SEQ ID NO: 46 is the putative leader region of β -secretase [1-21]~~[1-22]~~.

SEQ ID NO: 47 is the putative pre-pro region of β -secretase [22-45][~~23-45~~].

Please replace the paragraph beginning on page 14, line 7, with the following amended paragraph:

The term "fragment," when referring to β -secretase of the invention, means a polypeptide which has an amino acid sequence which is the same as part of but not all of the amino acid sequence of full-length β -secretase polypeptide. In the context of the present invention, the full length β -secretase is generally identified as SEQ ID NO: 2, the ORF of the full-length nucleotide; however, according to a discovery of the invention, the naturally occurring active form is probably one or more N-terminal truncated versions, such as amino acids 46-501 (SEQ ID NO:43), 22-501 (SEQ ID NO:66), 58-501 (SEQ ID NO:67) or 63-501 (SEQ ID NO:69); other active forms are C-terminal truncated forms ending between about amino acids 450 and 452. The numbering system used throughout is based on the numbering of the sequence SEQ ID NO: 2.

Please replace the paragraph beginning on page 14, line 16, with the following amended paragraph:

An "active fragment" is a β -secretase fragment that retains at least one of the functions or activities of β -secretase, including but not limited to the β -secretase enzyme activity discussed above and/or ability to bind to the inhibitor substrate described herein as P10-P4'staD->V (SEQ ID NO:72). Fragments contemplated include, but are not limited to, a β -secretase fragment which retains the ability to cleave β -amyloid precursor protein to produce β -amyloid peptide. Such a fragment preferably includes at least 350, and more preferably at least 400, contiguous amino acids or conservative substitutions thereof of β -secretase, as described herein. More preferably, the fragment includes active aspartyl acid residues in the structural proximities identified and defined by the primary polypeptide structure shown as SEQ ID NO: 2 and also denoted as "Active-D" sites herein.

Please replace the paragraph beginning on page 30, line 5, with the following amended paragraph:

The full-length open reading frame (ORF) of human β -secretase is described above, and its sequence is shown in FIG. 2A as SEQ ID NO: 2. However, as mentioned above, a further discovery of the present invention indicates that the predominant form of the active, naturally occurring molecule is truncated at the N-terminus by about 45 amino acids. That is, the protein purified from natural sources was N-terminal sequenced according to methods known in the art (Argo Bioanalytica, Morris Plains, NJ.). The N-terminus yielded the following sequence: EGDEEPEEPGRSGFSVEMVDNLRG... (SEQ ID NO: 55). This corresponds to amino acids 46-69 of the ORF-derived putative sequence. Based on this observation and others described below, the N-terminus of an active, naturally occurring, predominant human brain form of the enzyme is amino acid 46, with respect to SEQ ID NO: 2. Further processing of the purified protein provided the sequence of an internal peptide: IGFAVSACHVHDEFR ~~ISFAVSACHVHDEFR~~ (SEQ ID NO: 56), which is amino terminal to the putative transmembrane domain, as defined by the ORF. These peptides were used to validate and provide reading frame information for the isolated clones described elsewhere in this application.

Please replace the paragraph beginning on page 33, line 7, with the following amended paragraph:

Thus, a good candidate for crystallization is β -secretase 46-452 (SEQ ID NO: 58), since this is a form of the enzyme that (a) provides the predominant naturally occurring N-terminus, and (b) lacks the "sticky" transmembrane region, while (c) retaining β -secretase activity. Alternatively, forms of the enzyme having extensions that extend part of the way (approximately 10-15 amino acids) into the transmembrane domain may also be used. In general, for determining X-ray crystallographic coordinates of the ligand binding site, any form of the enzyme can be used that either (i) exhibits β -secretase activity, and/or (ii) binds to a known inhibitor, such as the inhibitor ligand P 10-P4'staD- > V, with a binding affinity that is at least 1/100 the binding affinity of P-secretase [46-501] (SEQ ID NO. 43) to P10-P4'staD- > V

(SEQ ID NO:72). Therefore, a number of additional truncated forms of the enzyme can be used in these studies. Suitability of any particular form can be assessed by contacting it with the P10-P4'staD- > V affinity matrix described above. Truncated forms of the enzyme that bind to the matrix are suitable for such further analysis. Thus, in addition to 46-452, discussed above, experiments in support of the present invention have revealed that a truncated form ending in residue 419, most likely 46-419 (SEQ ID NO:71), also binds to the affinity matrix and is therefore an alternate candidate protein composition for X-ray crystallographic analysis of β -secretase. More generally, any form of the enzyme that ends before the transmembrane domain, particularly those ending between about residue 419 and 452 are suitable in this regard.

Please replace the paragraph beginning on page 35, line 41, with the following amended paragraph:

According to one discovery of the present invention, the full-length β -secretase enzyme contains at least one transmembrane domain, and its purification is aided by the use of a detergent (Triton X-100). Membrane proteins can be crystallized intact, but may require specialized conditions, such as the addition of a non-ionic detergent, such as C₈G (8-alkyl- β -glucoside) or an n-alkyl-maltoside (C_nM). Selection of such a detergent is somewhat empirical, but certain detergents are commonly employed. A number of membrane proteins have been successfully "salted out" by addition of high salt concentrations to the mixture. PEG has also been used successfully to precipitate a number of membrane proteins (Ducruix, *et al.*, *supra*). Alternatively, as discussed above, a C-terminal truncated form of the protein that binds inhibitor but which lacks the transmembrane domain, such as β -secretase 46-452 (SEQ ID NO:58), is crystallized.

Please replace the paragraph beginning on page 38, line 8, with the following amended paragraph:

Various recombinant forms of the enzyme were produced and purified from transfected cells. Since these cells were made to overproduce the enzyme, it was found that the purification scheme described with respect naturally occurring forms of the enzyme (e. g.,

Example 5A) could be shortened, with positive results. For example, as detailed in Example 6, 293T cells were transfected with pCEKclone 27 (FIG. 12 and FIG. 13A-E) (SEQ ID NO:48) and Cos A2 cells were transfected with pCF β A2 using "FUGENE" 6 Transfection Reagent (Roche Molecular Biochemicals Research, Indianapolis, IN). The vector pCF was constructed from the parent vector pCDNA3, commercially available from Invitrogen, by inserting SEQ ID NO: 80 (FIG. 11A) between the HindIII and EcoRI sites. This sequence encompasses the adenovirus major late promoter tripartite leader sequence, and a hybrid splice created from adenovirus major late region first exon and intron and a synthetically generated IgG variable region splice acceptor.

Please replace the paragraph beginning on page 43, line 21, with the following amended paragraph:

The invention includes further cloning and expression of members of the aspartyl protease family described above, for example, by inserting polynucleotides encoding the proteins into standard expression vectors and transfecting appropriate host cells according to standard methods discussed below. Such expression vectors and cells expressing, for example, the human β -secretase enzyme described herein, have utility, for example, in producing components (purified enzyme or transfected cells) for the screening assays discussed in Part B, below. Such purified enzyme also has utility in providing starting materials for crystallization of the enzyme, as described in Section III, above. In particular, truncated form(s) of the enzyme, such as 1-452 (SEQ ID NO:59) and 46-452 (SEQ ID NO:58), and the deglycosylated forms of the enzyme described herein are considered to have utility in this regard, as are other forms truncated partway into the transmembrane region, for example ~~1-460 or 46-458~~ amino acid residues 1-460 or 46-458, respectively, in reference to SEQ ID NO:2.

Please replace the paragraph beginning on page 53, line 21, with the following amended paragraph:

In the case of β -secretase, knowledge of the amino acid sequence surrounding the cleavage site of APP and of the cleavage site of APP^{sw} has provided information for purposes of

setting up the phage display screening library to identify preferred substrates of β -secretase. As mentioned above, knowledge of the sequence of a particularly good peptide inhibitor, P10-P4staD- > V (SEQ ID NO:72), as described herein, provides information for setting up a "biased" library toward this sequence.

Please replace the paragraph beginning on page 55, line 4, with the following amended paragraph:

Part B, above, describes method of screening for compounds having β -secretase inhibitory activity. To summarize, guidance is provided for specific methods of screening for potent and selective inhibitors of β -secretase enzyme. Significantly, the practitioner is directed ~~to a~~ to a specific peptide substrate/inhibitor sequences, such as P10-P4'staD->V (SEQ ID NO:72), on which drug design can be based and additional sources, such as biased phage display libraries, that should provide additional lead compounds.

Please replace the paragraph beginning on page 59, line 8, with the following amended paragraph:

Poly A+ RNA from IMR human neuroblastoma cells was reverse transcribed using the Perkin-Elmer kit. Eight degenerate primer pools, each 8 fold degenerate, encoding the N and C terminal portions of the amino acid sequence obtained from the purified protein were designed (shown in Table 4; oligos 3407 through 3422) (SEQ ID NOS:3-21). PCR reactions were composed of cDNA from 10 ng of RNA, 1.5 mM $MgCl_2$, 0.125 μ l AmpliTaq Gold, 160 μ M each dNTP (plus 20 μ M additional from the reverse transcriptase reaction), Perkin-Elmer TAQ buffer (from AmpliTaq Gold kit, Perkin-Elmer, Foster City, CA), in a 25 μ l reaction volume. Each of oligonucleotide primers 3407 through 3414 was used in combination with each of oligos 3415 through 3422 for a total for 64 reactions. Reactions were run on the Perkin-Elmer 7700 Sequence Detection machine under the following conditions: 10 min at 95°C, 4 cycles of, 45°C annealing for 15 ~~seconds~~seconds, 72°C extension for 45 ~~second~~ seconds and 95°C denaturation for 15 seconds followed by 35 cycles under the same conditions with the exception that the annealing temperature was raised to 55°C. (The foregoing conditions are referred to

herein as "Reaction 1 conditions.") PCR products were visualized on 4% agarose gel (Northern blots) and a prominent band of the expected size (68 bp) was seen in reactions, particularly with the primers 3515-3518. The 68 kb band was sequenced and the internal region coded for the expected amino acid sequence. This gave the exact DNA sequence for 22 bp of the internal region of this fragment.

Please replace the paragraph beginning on page 59, line 26, with the following amended paragraph:

Additional sequence was deduced from the efficiency of various primer pools of discrete sequence in generating this PCR product. Primer pools 3419 to 3422 (SEQ ID NOS:15-18) gave very poor or no product, whereas pools 3415 to 3418 (SEQ ID NOS:11-14 respectively) gave robust signal. The difference between these pools is a CTC (3415 to 3418) (SEQ ID NOS:11-14) vs TTC (3419 to 3422) (SEQ ID NOS:15-18) in the 3' most end of the pools. Since CTC primed more efficiently we can conclude that the reverse complement GAG is the correct codon. Since Met coding is unique it was concluded that the following codon is ATG. Thus the exact DNA sequence obtained is:

CCC. GGC. CGG. AGG. GGC. AGC. TTT. GTG. GAG. ATG. GT (SEQ ID NO: 49)
encoding the amino acid sequence P G R R G S F V E M V (SEQ ID NO: 50). This sequence can be used to design exact oligonucleotides for 3 and 5' RACE PCR on either cDNA or libraries or to design specific hybridization probes to be used to screen libraries.

Please replace the paragraph beginning on page 60, line 11, with the following amended paragraph:

For generation of a probe, PCR reactions using oligonucleotides 3458 (SEQ ID NO:19) and 3469 (SEQ ID NO:21) or 3458 (SEQ ID NO: 19) and 3468 (SEQ ID NO: 20) (Table 4) can be carried out using the 23 RACE product, clone 9C7E.35 (30 ng, clone 9C7E.35 was isolated from origene library, see Example 2), or cDNA generated from brain, using the standard PCR conditions (Perkin-Elmer, rtPCR and AmpliTaq® Gold kits) with the following ~~following~~: 25 µl reaction volume 1.5 mM ~~MgCl₂~~ MgCl₂, 0.125 µl of AmpliTaq® Gold (Perkin-Elmer), initial 95° for 10 min to activate the AmpliTaq® Gold, 36 cycles of 65° for 15 sec, 72° for 45

sec, 95° for 15 sec, followed by 3 min at 72°. Product was purified on a Quiagen PCR purification kit and used as a substrate for randompriming to generate a radiolabelled probe (Sambrook, *et al.*, *supra*; Amersham RediPrime® kit). This probe was used to isolate full length close pCEK clone 27 shown in FIGS. 12 and 13 (A-E) (SEQ ID NO:48).

Please replace the paragraph beginning on page 61, line 3, with the following amended paragraph:

The fractions of transformed ~~E. Coli~~ *E. coli* were plated on Terrific Broth agar plates containing ampicillin and let grown for 18 hours. Each fraction yielded about 200,000 colonies to give a total of one million colonies. The colonies were then scraped from the plates and plasmids isolated from them in pools of approximately 70,000 clones/pool. 70,000 clones from each pool of the library was screened for the presence of the putative β -secretase gene using the diagnostic PCR reaction (degenerate primers 3411 (SEQ ID NO:7) and 3417 (SEQ ID NO:13) shown above).

Please replace the paragraph beginning on page 61, line 10, with the following amended paragraph:

Clones from the 1.5 kb pool were screened using a radiolabeled probe generated from a 390 b.p. PCR product generated from clone 9C7E.35. For generation of a probe, PCR product was generated using 3458 (SEQ ID NO:19) and 3468 (SEQ ID NO:20) as primers and clone 9C7E.35 (30 ng) as substrate.

Please replace the paragraph beginning on page 61, line 14, with the following amended paragraph:

PCR product was used as a substrate for random priming to generate a radiolabeled probe. 180,000 clones from the 1.5 kb pool (70,000 original clones in this pool), were screened by hybridization with the PCR probe and 9 positive clones identified. Four of these clones were isolated and by restriction mapping these appear to encode two independent clones of 4 to 5 kb (clone 27) and 6 to 7 kb (clone 53) length. Sequencing of clone 27 verified

that it contains a coding region of 1.5 kb. FIG. 13 (A-E) shows the sequence of pCEK clone27 (clone 27) (SEQ ID NO:48).

Please replace the paragraph beginning on page 62, line 9, with the following amended paragraph:

94 wells from the master plate were screened using PCR. The Reaction 1 Conditions described in Example 1, above, were followed, using only primers 3407 (SEQ ID NO:3) and 3416 (SEQ ID NO:12) with 30ng of plasmid DNA from each well. Two pools showed the positive 70bp band. The same primers and conditions were used to screen 1µl *E. coli* from each well of one of the subplates. *E. coli* from the single positive well was then plated onto LB/amp plates and single colonies screened using the same PCR conditions. The positive clone, about 1Kb in size, was labeled 9C7E.35. It contained the original peptide sequence as well as 5' sequence that included a methionine. The 3' sequence did not contain a stop codon, suggesting that this was not a full-length clone, consistent with Northern blot data.

Please replace the paragraph beginning on page 63, line 11, with the following amended paragraph:

For reverse transcription polymerase chain reaction (RT-PCR), two partially degenerate primer sets used for RT-PCR amplification of a cDNA fragment encoding this peptide. Primer set 1 consisted of DNA's #3427-3434 (SEQ ID NOS:22-29 respectively), the sequences of which are shown in Table 5, below. Matrix RT-PCR using combinations of primers from this set with cDNA reverse transcribed from primary human neuronal cultures as template yielded the predicted 54 bp cDNA product with primers #3428 + 3433 (SEQ ID NOS:23-28 respectively). All RT-PCR reactions employed 10-50 ng input poly-A+ RNA equivalents per reaction, and were carried out for 35 cycles employing step cycle conditions with a 95°C denaturation for 1 minute, 50°C annealing for 30 sec, and a 72°C extension for 30 sec.

Please replace the paragraph beginning on page 63, line 20, with the following amended paragraph:

The degeneracy of primers #3428 + 3433 (SEQ ID NOS:23-28) was further broken down, resulting in primer set 2, comprising DNAs #3448-3455 (SEQ ID NOS:30-37) (Table 5). Matrix RT-PCR was repeated using primer set 2, and cDNA reverse transcribed from poly-A+ RNA from IMR-32 human neuroblastoma cells (American Type Culture Collection, Manassas, VA), as well as primary human neuronal cultures, as template for amplification. Primers #3450 (SEQ ID NO:32) and 3454 (SEQ ID NO:36) from set 2 most efficiently amplified a cDNA fragment of the predicted size (72 bp), although primers 3450+3453 (SEQ ID NOS:32 and 35), and 3450+3455 (SEQ ID NOS:32 and 37) also amplified the same product, albeit at lower efficiency. A 72 bp PCR product was obtained by amplification of cDNA from IMR-32 cells and primary human neuronal cultures with primers 3450 (SEQ ID NO:32) and 3454 (SEQ ID NO:36).

Please replace the paragraph beginning on page 63, line 30, with the following amended paragraph:

Internal primers matching the upper (coding) strand for 3' Rapid Amplification of 5' Ends (RACE) PCR, and lower (non-coding) strand for 5' RACE PCR were designed and made according to methods known in the art (e.g., Frohman, M. A., M. K. Dush and G. R. Martin (1988). "Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene specific oligo-nucleotide primer." Proc. Natl. Acad. Sci. U.S.A. **85**(23): 8998-9002.) The DNA primers used for this experiment (#3459 & #3460) (SEQ ID NOS:38 and 39) are illustrated schematically in FIG. 4, and the exact sequence of these primers is presented in Table 3. These primers can be utilized in standard RACE-PCR methodology employing commercially available templates (e.g. Marathon Ready cDNA®, Clontech Labs), or custom tailored cDNA templates prepared from RNAs of interest as described by Frohman et al. (ibid.).

Please replace the paragraph beginning on page 64, line 8, with the following amended paragraph:

In experiments carried out in support of the present invention, a variation of RACE was employed to exploit an IMR-32 cDNA library cloned in the retrovirus expression vector pLPCXlox, a derivative of pLNCX. As the vector junctions provide unique anchor sequences abutting the cDNA inserts in this library, they serve the purpose of 5' and 3' anchor primers in RACE methodology. The sequences of the specific 5' and 3' anchor primers we employed to amplify β -secretase cDNA clones from the library, primers #3475 (SEQ ID NO:40) and #3476 (SEQ ID NO:41), are derived from the DNA sequence of the vector provided by Clontech Labs, Inc., and are shown in Table 3.

Please replace the paragraph beginning on page 64, line 16, with the following amended paragraph:

Primers #3459 (SEQ ID NO:38) and #3476 (SEQ ID NO:41) were used for 3' RACE amplification of downstream sequences from our IMR-32 cDNA library in the vector pLPCXlox. The library had previously been sub-divided into 100 pools of 5,000 clones per pool, and plasmid DNA was isolated from each pool. A survey of the 100 pools with the primers identified as diagnostic for presence of the β -secretase clone, according to methods described in Example 1, above, provided individual pools from the library for RACE-PCR. 100 ng template plasmid from pool 23 was used for PCR amplification with primers 3459+3476 (SEQ ID NOS:38 and 41 respectively). Amplification was carried out for 40 cycles using ampli-Taq Gold®, under the following conditions: denaturation at 95°C for 1 min, annealing at 65°C for 45 sec., and extension at 72°C for 2 min. Reaction products were fractionated by agarose gel chromatography, according to methods known in the art (Ausubel; Sambrook).

Please replace the paragraph beginning on page 64, line 27, with the following amended paragraph:

An approximately 1.8 Kb PCR fragment was revealed by agarose gel fractionation of the reaction products. The PCR product was purified from the gel and subjected

to DNA sequence analysis using primer#3459 (SEQ ID NO:38). The resulting sequence, designated 23A, and the predicted amino acid sequence deduced from the DNA sequence are shown in FIG. 5. Six of the first seven deduced amino-acids from one of the reading frames of 23A were an exact match with the last 7 amino-acids of the N-terminal sequence determined from the purified protein, purified and sequenced in further experiments carried out in support of the present invention, from natural sources.

Please replace the paragraph beginning on page 76, line 26, with the following amended paragraph:

All manipulations were carried out at room temperature. 12.5 ml of 80% slurry of NHS-Sepharose (i.e. 10 ml packed volume; Pharmacia, Piscataway, NJ) was poured into a Bio-Rad EconoColumn (BioRad, Richmond, CA) and washed with 165 ml of ice-cold 1.0 mM HCl. When the bed was fully drained, the bottom of the column was closed off, and 5.0 ml of 7.0 mg/ml P10-P4'sta(D->V) peptide (SEQ ID NO:72) (dissolved in 0.1 M HEPES, pH 8.0) was added. The column was capped and incubated with rotation for 24 hours. After incubation, the column was allowed to drain, then washed with 8 ml of 1.0 M ethanolamine, pH 8.2. An additional 10 ml of the ethanolamine solution was added, and the column was again capped and incubated overnight with rotation. The column bed was washed with 20 ml of 1.5 M sodium chloride, 0.5 M Tris, pH 7.5, followed by a series of buffers containing 0.1 mM EDTA, 0.2% Triton X-100, and the following components; 20 mM sodium acetate, pH 4.5 (100 ml); 20 mM sodium acetate, pH 4.5, 1.0 M sodium chloride (100 ml); 20 mM sodium borate, pH 9.5, 1.0 M sodium chloride (200 ml); 20 mM sodium borate, pH 9.5 (100 ml). Finally, the column bed was washed with 15 ml of 2 mM Tris, 0.01% sodium azide (no Triton or EDTA), and stored in that buffer, at 4°C.

Please replace the sequence listing, submitted July 13, 2001, with the paper copy of the substitute sequence listing (41 pages, sequentially numbered 1-41) submitted herewith.